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Hypothesis Paper

MECHANISM OF ANTIBACTERIAL ACTION: ELECTRON TRANSFER AND OXY RADICALS

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Abstract—Most of the main categories of bactericidal agents, namely, aliphatic and heterocyclic nitro compounds, metal derivatives and chelators, quinones, azo dyes, and iminium-type ions, are proposed to exert their action by a unified mechanism. The toxic effect is believed to result generally from the catalytic production of reactive oxygen radicals that usually arise via electron transfer. Cyclic voltammetry was performed on a number of these agents. Reductions were for the most part reversible, with potentials in the favorable range of −0.20 to −0.58 V.

Keywords—Antibacterial action, Nitro heterocycles, α-Halonitro compounds, Metal derivatives and chelators, Quinones, Azo dyes, Iminium ions

INTRODUCTION

...radical generation and electron transfer (ET) phe...ena are being increasingly implicated as mechan...pathways for a variety of xenobiotics and in human ...ses.[1-9] In our laboratory, application of this gen...theme has been made to carcinogens,[10] anticancer ...s,[11-14] antimalarials,[15] benzodiazepines,[16] phen...dine (PCP),[17] nicotine,[17] spermine,[17] 1-methyl-4-...yl-1,2,3,6-tetrahydropyridine (MPTP),[18] me...ics,[19] and heterocyclic di-*N*-oxides.[20,21] There ar...to be several main classes of CT agents: quinone ... and precursors, metal (for example, Cu and Fe) ...lexes, $ArNO_2$, and iminium salts.[22] According to ...nifying hypothesis, the ultimate form of the drug ...tively binds to a receptor, for example, bacterial ... and effects catalytic electron transfer to oxygen, ...cing toxic oxy radicals that destroy the cell. Al...tively, there may be interference with normal ...ron transport chains.

...e natural defense against invading microorga...s involves the use of reactive oxygen species gen...d by neutrophilic granulocytes during phagocy...[23-25] The rapid uptake of oxygen results in the formation of superoxide, hydrogen peroxide, and hydroxyl radicals. Other species that may be involved are HOCl and singlet oxygen. Hypochlorous acid can react readily with cellular constituents to generate labile *N*-chloro entities. It is quite significant that this scenario represents the normal response that has been found by evolutionary development to be most effective. Similar operation by various antibacterial drugs would comprise a reasonable working hypothesis.

A number of peroxides are reported to display antibacterial activity. Evidence shows that exogenous hydrogen peroxide is able to destroy bacteria.[23,26] There was a marked increase in killing efficiency, presumably because of the Fenton reaction, when the iron content of the system increased. Generation of oxy radicals may also account for other biological effects of this peroxide, for example, carcinogenic,[10] mutagenic,[27] and anticancer.[11-14] Ethyl hydroperoxide, which is not decomposed by catalase, was more effective than the parent as a bactericide against *Escherichia coli*.[28] Cyclic peroxides can act as antibacterial,[29] antimalarial,[15] and antifungal[30] agents. ESR studies with some members demonstrated the ability to function as precursors of hydroxyl radicals.[31] Peracetic acid has been considered for use as a sterilant.[32] The diverse peroxides can serve as precursors of oxy radicals, similar to the phagocytic process.

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A related category consists of *N*-chloro derivatives of sulfonamides, cyclic imides, and amidines.[32] Familiar examples are chloramine T, dichloramine T, and halazone. Although it is known that chlorine can be transferred to a nitrogen-containing cellular receptor, the precise mechanism of bioaction is unresolved. These substances represent analogues of the *N*-chloro derivatives generated during phagocytosis.

Although individual groups of antibacterial agents have been rationalized mechanistically,[32] there has been no report of a broad, detailed, systematic approach. Quite some years ago, suggestions were made that charge transfer and oxidative stress might well play a role in the action of some medicinals.[33-35] We now propose a unifying theme based on the ET–oxy radical concept that encompasses a large variety of bactericides, including metal derivatives of mercury (merbromin, merthiolate), metal chelators (nalidixic acid and oxine), nitroheterocycles (nitrofurazone, nitrofurantoin), nitroaliphatics (chloropicrin, bronopol), quinones (halogenated *o*-naphthoquinones), azo dyes (scarlet red), and iminium species (triarylmethane dyes, heterocyclic di-*N*-oxides, and heterocyclic salts). These represent almost all of the major categories discussed in *Burger's Medicinal Chemistry*.[32] Our experimental goal was to determine the reduction potential and reversibility for the various classes in order to obtain evidence concerning the feasibility of catalytic electron transfer in vivo. In some cases, literature data provide the requisite base for incorporation within the theoretical framework. Related considerations are also discussed.

MATERIALS AND METHODS

Materials

Chemicals and antibacterial agents were obtained from the Aldrich Chemical Company (Milwaukee, WI) or the Sigma Chemical Company (St. Louis, MO), except $CuCl_2 \cdot 2H_2O$ (MCB, Norwood, OH), $FeCl_3$ (Baker and Adamson, Morristown, NJ), chloropicrin (Eastman Kodak, Rochester, NY), and silver sulfadiazine (Polysciences, Warrington, PA). 6-Bromo-1,2-naphthoquinone was prepared using Fremy's salt by published methods[36] (m.p. 164–166°C [dec], lit. 167°C). The infrared spectrum (Perkin Elmer model 735B) displayed the reported peaks. Elemental analysis for $C_{10}H_5BrO_2$ was as follows: calculated: C, 50.7; H, 2.1; found: C, 50.4; H, 2.2.

The electrolyte used in the nonbuffered electrochemical studies was tetraethylammonium perchlorate (0.1 M) (G. F. Smith, Columbus, OH). Dimethylformamide (DMF) (Aldrich) was obtained in the highest available purity. Absolute ethanol (U.S. Industrial, Tuscola, IL) was used to prepare the aqueous solutions; pH 7.0 aqueous buffer (0.1 M KH_2PO_4/0.1 M NaOH) was used for some measurements.

Methods

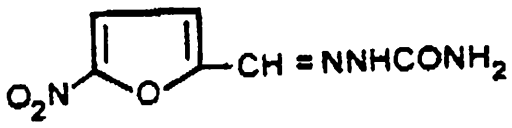
The cyclic voltammetric measurements were performed at ambient temperature with a Princeton Applied Research Corporation (PARC) model 174A polarographic analyzer connected to a Hewlett Packard model 7035B X-Y recorder. The scan rates generally ranged from 20 to 200 mV/s. Solutions were saturated for 15 min with prepurified nitrogen (30 min for metal complexes) that was passed through an oxygen-scrubbing system. The electrodes consisted of either a platinum flag (Sargent-Welch, Skokie, IL) or a hanging mercury drop (HMDE) working electrode, with a platinum wire as the counter. The reference was a saturated calomel electrode (SCE) (Corning). Observed potentials (our work and literature values) were converted to the normal hydrogen electrode (NHE) by the addition of 0.24 V to the SCE values. The reported data are the average of two or more measurements involving fresh solutions. The following equations were used for the half-wave potentials, the differences in potentials, and current function: $E_{1/2} = (E_{pc} + E_{pa})/2$, $\Delta E_p = |E_{pa} - E_{pc}|$, $E_{pp/2} = |E_{pc} - E_{pc/2}|$, and $CF = i_p/V^{1/2} \times C$.

RESULTS AND DISCUSSION

Nitro compounds

1. *Nitro heterocycles.* This chemotherapeutic category encompasses mainly nitro derivatives of furan and imidazole.[32] Specific examples of the furan class include nitrofurazone, 1, and nitrofurantoin, 2.

Electrochemical studies were performed on both 1 and 2. The results are presented in Table 1. Compound 1 undergoes a one-electron reduction (*CF* ratio 0.88; benzil[42] as reference) with $E_{1/2} = -0.67$ V, in agreement with the literature (Table 1). Reduction to a radical anion is in agreement with prior studies of nitrofurans in aprotic solvent.[37] The reduction was quasi-reversible and diffusion controlled, as characterized by the ΔE_p values, 70–90 mV, and the constant current function. The nitro anion formed is quite stable with an i_{pc}/i_{pr} value of 0.97 at 100 mV/s sweep rate. Nitrofurantoin, 2, reduced in two waves: the difference in cathodic peak potentials was 110 mV at the sweep



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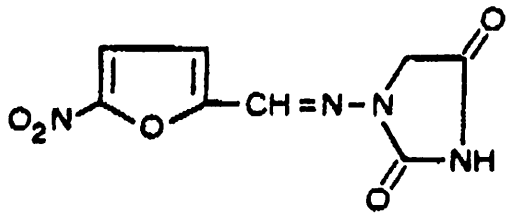


(2)

ate of 0.1 V/s. The first wave, $E_{p/2}$ = −0.66 V, was reversible, with $E_{pp/2}$ of 80 mV. The *CF* ratio (0.98) indicates the transfer of one electron. The current function decreased as the scan rate was lowered, suggesting that reduction was followed by another chemical reaction. The second wave was quasi-reversible with a 30-mV change in E_p upon a decade change in sweep rate (−0.74 V at 20 mV/s). The $E_{1/2}$ was −0.73 V with ΔE_p of 80 mV. These reductions may involve both nitro and conjugated imine. The value for the one-electron reduction of 2-nitrofuran in DMF is −0.76 V.[37] The potentials in our study are more positive because of increased conjugation.

Reductions of 1 and 2 have also been examined in buffered media. Nitrofurazone exhibited two irreversible waves (−0.23 and −0.97 V) at pH 8.6.[43] The process involved the reduction of both nitro and imine moieties. Another investigation[44] resulted in values of −0.04 and −0.02 V for 1 and 2, respectively. An earlier study reported multiple waves for 2 in aqueous buffer.[45] The proposed pathway involved reductive fission of the N–N bond. The prior data are difficult to compare to ours because of differences in conditions, for example, solvent.

The mechanism of action for this general class has been reviewed recently.[5,46–48] A correlation is observed between electron affinity and radio-sensitization, hypoxic cell toxicity, and chronic aerobic toxicity, suggesting that redox processes play an important role. In an aerobic environment the intermediate nitro radical anion readily conveys an electron to oxygen. For example, nitrofurantoin stimulated the uptake of oxygen in hepatic incubations, which was partially reversed by superoxide dismutase (SOD) and catalase; this suggested the presence of superoxide and H_2O_2.[49] Increased oxygen consumption has also been observed for nitrofurazone. Activated oxygen may also be responsible for toxic effects, such as pulmonary edema and fibrosis, that may occur during nitrofurantoin therapy.[49] In other cases, including anaerobic conditions, the radical anion exerts its effect by another route, presumably interference with normal electron transport. A serious problem with these types is acute toxicity, usually associated with mutagenesis or carcinogenesis. One can speculate that both the favorable effect and the liabilities might be intimately related to the ease of electron transfer. Prior reviews have discussed ESR of the intermediate nitro radical anions.[5,49]

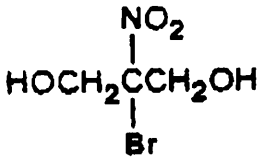
2. *α-Halonitro aliphatics.* The common structural theme is the presence of the α-halonitro moiety along with two other electron withdrawing substituents.[32] Members are exemplified by bronopol, 3, and chloropicrin, 4. Compound 4 is a sterilant.

Bronopol underwent irreversible reduction with the most positive peak at E_p = −0.56 V (Table 1). The plot of the peak current versus the square root of the sweep rate was linear and passed through the origin. $E_{pp/2}$ values varied from 180 to 260 mV for the scan rates employed. The E_p changed 190 mV with a tenfold increase in sweep rate. The *CF* ratio of bronopol versus benzil as the reference gave a value of 0.89, indicating that only one electron was transferred. The absence of an oxidation peak is rationalized by the participation of a chemical reaction after initial reduction, for ex-

Table 1. Electrochemical Characteristics of Nitro Derivatives of Heterocyclic and Aliphatic Compounds

Substrate	Reduction Potential (V)	i_{pa}/i_{pc}	*CF* ratio[a]	Reference
1[b]	−0.67[c]	0.97	0.88	—
1[d]	−0.68	—	—	—
2[b,e]	−0.66[f]	—	0.98	—
2[b,g]	−0.73[c]	—	—	—
2-Nitrofuran	−0.76	—	—	37
3[b]	−0.56[f]	—	0.89	—
3	−0.10	—	—	37
4[b]	−0.71[f]	—	1.99	—
2-Bromo-2-nitropropane	−0.4	—	—	38
2-Chloro-2-nitropropan-1,3-diol	−0.54	—	—	39
Nitroethane	−1.25	—	—	40

[a] *CF* ratio = CF_{exptl}: CF_{std}. = $A\,(V\,s)^{1/2}C$ = 16.27 (0.5 mM, Pt, DMF). 0.185 (0.5 mM, HMDE, DMF). [b] 100 mV/s, tetraethylammonium perchlorate (0.1 M), substrate (0.5 mM), DMF, versus NHE, Pt electrode. [c] Reversible. [d] Ref. 41. [e] First wave. [f] Irreversible. [g] Second wave.



(3)

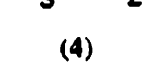
ample, dimerization. The reduction of 3 is similar to that of 2-bromo-2-nitropropane in CH_3CN, E_p of about -0.4 V.[38] The reaction involved one-electron reduction with subsequent loss of bromide ion. The ensuing products that arise from hydrogen abstraction or dimerization might function as stable ET catalysts. In a prior study, compound 3 in aqueous solution gave irreversible reduction at -0.10 V.[39] Electrolysis yielded 2-nitropropane-1,3-diol, which has also been observed as a metabolite of bronopol in rats and dogs.[50]

Chloropicrin exhibited irreversible reduction with E_p of -0.71 V (Table 1). A linear plot passing through the origin was obtained for the peak current versus the square root of the sweep rate. The $E_{pp/2}$ value at 100 mV/s was 230 mV. The reduction potential changed 110 mV upon a tenfold increase in sweep rate. Comparison of the current function with benzil gave a value of 1.99, indicating the transfer of two electrons. The number of electrons involved in the reaction of 4 is difficult to explain on the basis of the discussion for 3. However, a possible rationale entails formation of a carbene intermediate. Such a process was observed for the two-electron reduction of gem-dihalides in aprotic media.[51] Chloropicrin undergoes two-electron reduction in aqueous buffer,[52] yielding an $E_{1/2}$ value of $+0.30$ V that is invariant with pH.

The literature contains an appreciable amount of work on the electroreduction of aliphatic nitro compounds. The $E_{1/2}$ values range from about -1.33 V for nitroethane, 1-nitropropane, and 1-nitrobutane[40] to -1.40 V for 2-nitropropane[53] in DMF. The $E_{1/2}$ (-0.64 V) for nitromethane is the same as for 1-nitropropane in pH 7.0 buffer.[54] Electron-withdrawing groups, such as those in 3 and 4, should make the reduction potential more positive.

In relation to more detailed mechanistic considerations, the α-halonitro-alkanes readily accept an electron to yield a radical anion that eliminates halide.[38,55] The resulting delocalized radical could dimerize or combine with oxygen in a process leading to various reactive intermediates. Alternatively, uptake of another electron would generate the corresponding anion, which might abstract a proton or eliminate a remaining α-halogen.

Significant to our theme is the comparison of reduction potentials with activity for bronopol and its analogues. For example, the chloro counterpart of 3 exhibits a larger minimum inhibitory concentration toward bacteria than bronopol;[56] it is also more difficult to reduce,[39] -0.54 V versus -0.10 V for 3. 2-Bromo-2-nitropropane ($E_{1/2} \sim -0.4$ V) and 2-chloro-2-nitropropane ($E_{1/2} \sim -0.96$ V) show a similar relationship between $E_{1/2}$[38] and activity.[56] Reduction potential may also play a role in the antimicrobial activity of α-substituted 5-nitro-1,3-dioxanes (analogues of 3). Replacement of bromine by chlorine or hydrogen resulted in marked reduction in activity.[57] Additional evidence is available that sheds light on the mode of action. The biocidal effects of bronopol are inhibited by thiols.[32] Our ET-oxy radical hypothesis is in accord with this observation. It is significant that prior investigators have also proposed involvement of an oxidative mechanism.[56] However, their suggestion for the lethal action entailed oxidative conversion of enzymatic mercapto groups to disulfides. Inhalation of chloropicrin produced edema and lesions in the lungs of exposed animals.[58,59] The toxicity toward *Sitophilus granarius* and *Tenebroides mauritanicus* was potentiated by oxygen.[60] Compound 4 is an indirect, as well as a weak direct-acting, mutagen.[61]



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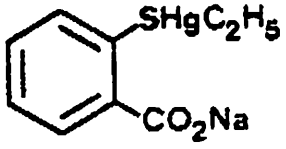
Metal-containing drugs

Metal species are known to elicit a variety of physiological responses. Specific chemical reactions pertinent to our approach that have been observed include oxy radical formation[4,62] and DNA strand cleavage.[63] The formation of complexes with DNA has been reported in some cases.[63,64] Much of the prior work is related to the cancer[10] and anticancer areas.[11] Of the various heavy metals employed over the years, mercury and silver remain the only ones widely used in the treatment or prevention of microbial disease.[32] The derivatives of heavy metals examined in this study include thiomersol (5, merthiolate), merbromin (6, mercurochrome), and silver sulfadiazine, 7.

Compound 5 reduced with $E_{1/2} = -0.50$ V in a process that was 44% reversible ($i_{pa}/i_{pc} = 0.44$) at 100 mV/s in pH 7 aqueous buffer (Table 2). The ΔE_p was



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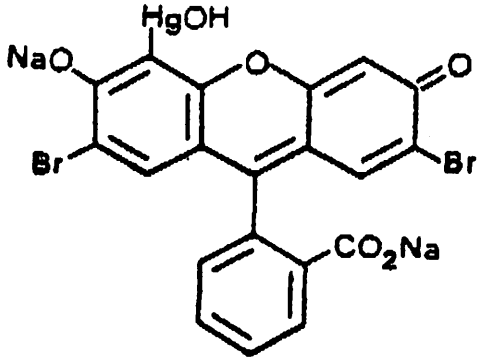

(6)

75 mV. Irreversibility pertained at slower scan rates. Diffusion control was evidenced by the constant CF value. A second peak was observed at −1.03 V. Upon a change of cathode to platinum, no reduction was observed in the buffer. The results for **5** are in agreement with prior studies.[65,66] The first wave varied with pH, $-E_{1/2} = 0.18 + 0.05$ pH;[65] the second wave, $E_{1/2} = -0.96$, was invariant with pH. The first step resulted in the formation of the alkylmercury radical,[65] which is known to arise from the reduction of organomercuric halides.[67]

Merbromin, **6**, reduced in two irreversible steps, the first with $E_p = -0.54$ V (Table 2). The process was diffusion-controlled. Irreversibility was supported by an $E_{p/2}$ value of 90–100 mV. Lower diffusion is expected for **6** due to its size, which should result in less current being passed. The maximum current for the first peak was approximately one-half that observed for **5**. The second peak occurred at $E_p = -0.74$ V. Drug **6** also gave no reduction in aqueous buffer with platinum as the working electrode, in line with other electrochemical studies involving mercury compounds.[66] Apparently, merbromin has not been extensively studied electrochemically. Page and Waller[66] reported no reduction in 1.0 M HCl. The $E_{1/2}$ for fluorescein in pH 6.25 buffer is −0.73 V[65] (no reference electrode given); the quinone methide form exists in neutral alcohol solution.[70]

Several lines of evidence are in agreement with the thesis that the toxic action of mercury compounds may arise from oxy radical generation via electron transfer. Thiomersol caused hemolysis, which was decreased by

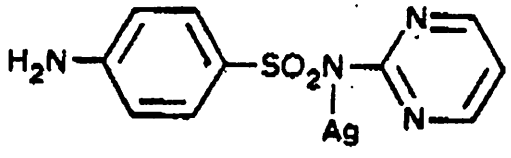

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Table 2. Reduction Potentials of Metal Derivatives[a]

Substrate	Electrode	Reduction Potential (V)	i_a/i_c
5[b]	HMDE	−0.50[c]	0.44
5[b]	Pt	—[d]	—
6[b]	HMDE	−0.54[c]	—
6[b]	Pt	—[d]	—
7[b]	HMDE	—[f]	—
Silver protein[b]	HMDE	—[d]	—
8 · $CuCl_2$[g]	Pt	−0.23[e]	—
8 · $FeCl_3$[g]	Pt	−0.21[e]	~1

[a] 100 mV/s, tetraethylammonium perchlorate (0.1 M, except in buffer), substrate (0.5 mM), versus NHE.
[b] pH 7.0 buffer (0.1 M KH_2PO_4/0.1 M NaOH).
[c] Quasi-reversible.
[d] No reduction before background.
[e] Irreversible.
[f] Insoluble.
[g] 50% aqueous ethanol.

DMSO,[71] a well-known oxy radical scavenger.[72] A number of fluorescent dyes act as hemolysins when irradiated with UV light or in darkness when present in high concentrations, or in combination with H_2O_2.[73] Oxidative stress is believed to be responsible for hemolysis by various antimalarial agents.[15] Thiomersol was observed to increase oxygen uptake in *Trichophyton*.[74] Organic mercury compounds are known to be metabolized to inorganic derivatives.[75] Mercuric chloride causes extensive DNA breakage, presumably by an oxygen-stress mechanism.[76,77] Lipid[78] and membrane[79] oxidation have been observed. Several common antioxidants, including Se and vitamin E, reduce the effects of Hg intoxication.[75]

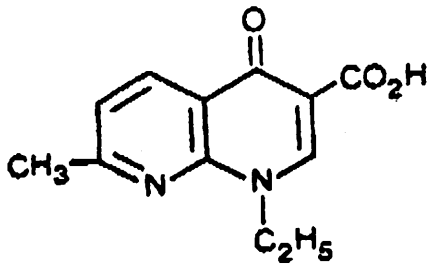
Prior rationale for the mode of action has involved the binding of mercury derivatives to the thiols of cellular proteins.[32,75] Mercury may form complexes with DNA.[64] The crystal structure of an unusual methyl mercury-adenine complex has been published recently.[80]

We were unable to obtain data for compound **7** and silver protein because of insolubility, which is not surprising since the drugs may be in a colloidal state. In vivo, metabolism could presumably take place, resulting in a form that can bind to DNA.[64] Ingestion of silver ion or colloidal silver produces liver necrosis in rats deficient in vitamin E.[75]

Ag ingestion

Metal chelators

1. *Quinolones.* Nalidixic acid, **8**, the most prominent member in this class, has received considerable attention.[32] It is used in the treatment of urinary tract infections and is particularly active against gram-negative organisms. Several reviews deal with structure activity relationship (SAR) and mode of action.[32,81,82] Various mechanisms have been proposed; recent at-



(8)

tention has been focused on coordination with metals. The conclusion was drawn that Cu(II) and/or Fe(II) are involved in the in vivo activity.[81]

Since the bactericidal activity may be associated with the formation of a 1:1 (8:metal) complex, we performed electrochemical studies on solutions of the sodium salt of nalidixic acid and $CuCl_2 \cdot 2H_2O$ or $FeCl_3$ at the 1:1 molar ratio in 50% aqueous ethanol. The results are summarized in Table 2. Sodium nalidixate produced no reduction before −0.8 V. Cupric chloride gave two irreversible reduction peaks, at +0.13 and −0.02 V. With equimolar concentrations of metal and 8 (Na salt), a broad irreversible peak with E_p of −0.23 V and a small peak at −0.01 V were observed. It is likely that the minor peak is due to uncomplexed copper. The E_p for the complex was −0.08 V at a sweep rate at 20 mV/s, while at 200 mV/s it was −0.30 V. Ferric chloride gave irreversible reduction, E_p = −0.59 V, with an anodic wave at +0.16 V. A small cathodic peak was present at +0.06 V with a current of about 25% of that at −0.59 V. The 1:1 solution of iron and 8 (Na salt) resulted in a quasi-reversible reduction at −0.21 V with ΔE_p of about 190 mV. The peak at about +0.1 V was also observed. The i_{pa}/i_{pc} ratio was about 1 at 100 mV/s, indicating the formation of a stable reduction product.

Evidence that a metal chelate of 8 may act as a potent ET agent was obtained from in vitro studies, indicating an increase in the ET rate from $FeSO_4$ to cytochrome *c*.[81] An intriguing relationship exists between bactericidal effect and concentration.[83] The activity is decreased by increasing levels of the drug, similar to the oxine case. A possible rationale is that some uncomplexed positions must be available on the metal to permit coordination with the active site, for example, DNA. Single-strand scission in DNA has been observed in the presence of 8.[84] These breaks are commonly associated with the formation of oxy radicals.[5,7] However, the potency toward *Plasmodium falciparum*, a malarial parasite sensitive to oxygen tension, was associated with low oxygen concentration.[85] Alternatively, activity may derive from the free form of 8. Recently, its one-electron reduction potential (−0.87 V at pH 6.4) was reported.[86]

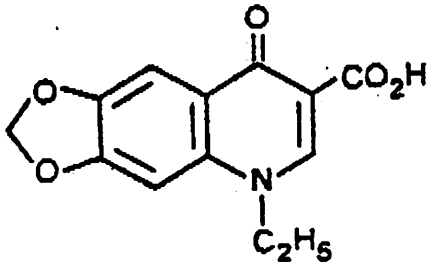
Other mechanisms proposed for the action of 8[81] include inhibition of RNA synthesis, DNA intercalation, and inhibition of DNA gyrase. However, it has been observed that an analogue of 8 does not bind to gyrase, but rather to DNA.[87] There is selective binding in vitro to guanine of DNA in the presence of a stoichiometric equivalent of the metal ion.[81]

Another member is oxalinic acid, 9, which displays an antibacterial spectrum similar to that of 8, but which is 2–4 times as potent.[32] Since metabolic studies reveal conversion to the corresponding catechol derivative, it is reasonable to expect subsequent facile oxidation to the *o*-quinone.[88] Hence, in vivo transformations of 9 could result in two electroactive sites, namely quinone and metal complex.

2. *8-Hydroxyquinoline* (*oxine*). The 8-hydroxyquinolines (antibacterial and antifungal)[32] can be included in this division, since they readily form metal complexes.[89] Of the various quinolinols, the 8-isomer is uppermost in its ability to chelate biochemically important metals. The physiological effects have been attributed to the generation of such coordination compounds. Iron and copper are known to play important roles with both endogenous and exogenous compounds, entailing oxy radical formation in some cases.[3,10,11] Significantly, data indicate that either the Cu(I) or Cu(II) form of related complexes might engage in ET.[90]

The Fe(III) 1:1 complex with oxine reduces at +0.52 V,[89] while the reduction potential for the 1:2 Cu(II) complex varies with pH, $E_{1/2}$ = 0.12 − 0.058 pH.[91] Iron oxine complexes catalyze the oxidation of thiols in nucleoproteins,[89] while the copper complex is toxic to algae in seawater.[92] The mechanism may be due to the formation of activated oxy species.

3. *Others*. Biguanides can be similarly classified. Chlorhexidine, 10, a widely used topical antiseptic, serves to illustrate.[32] Informative mechanistic studies have been carried out on the antimalarial counterparts,



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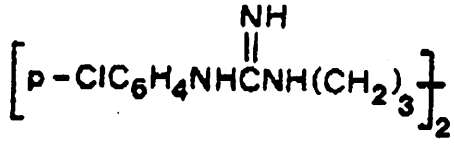

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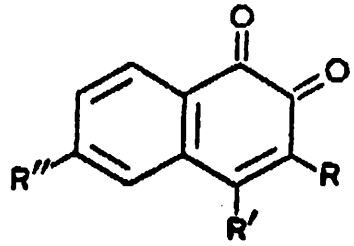
or example, chlorguanide (proguanil, paludrine). Although evidence has been found indicating the importance of metabolism, the conclusion was drawn from other studies that the activity against primate malaria must be attributed in part to the parent drug.[93] In prior electroreduction investigations, the indicated values were reported: −0.85 V from extrapolation to pH 0,[94] −1.38 (pH 7.05),[95] −1.38 V (pH 6.04),[96] and greater than 1.8 V in acid,[15] too negative to be operative in vivo. Biguanides coordinate readily with certain metal ions.[97,98] A value of −0.24 V for the reduction potential was obtained by other investigators using the Cu(II) complexes.[99] Hence, the general mechanistic scenario outlined for the other classes could also be applied here. The related amidines[92] may also operate in this manner. Additional antibacterial drugs that can chelate are[69] salicylic acid, hexachlorophane, antimycin, 2,2′-bipyridyl, 1,10-phenanthroline, dithiocarbamates, and oxides of pyridine, quinoline, or benzoquinoline with a coordinating group in the 2-position.

Quinones and phenols

Quinone antibiotics have found widespread application in recent years in the treatment of malignancy.[46,49,100,101] Extensive studies have principally involved anthracyclines, mitomycins, streptonigrin, and streptomycins. The toxicity, found to be oxygen-dependent,[100] apparently results from redox cycling of the quinone. Initial metabolic reduction to the semiquinone intermediate, which can evidently bind to DNA,[102] is an essential step.[101] The overall process has been designated site-specific free radical generation.[103] Inhibition of the rate of DNA scission was observed in added catalase, SOD, and free radical scavengers.[101] However, Adriamycin bound to DNA is unable to participate in redox reactions.[104] Strand scission can occur in the absence of binding. Alternatively, the ultimate agent may be a metal complex.[88] Electronic structures related to quinones are found in some other bactericidal agents,[105] for example, triarylmethane dyes and mercurochrome.

Recently, a number of halogen-substituted 1,2-naphthoquinones, **11** (R=R″=Br; R′=H; R=Cl, H, Br) were shown to possess antibacterial activity.[106] To determine the possible involvement in ET, we synthesized a congener, namely, 6-bromo-1,2-



(11)

R=R′=H, R″=Br

(11a)

naphthoquinone (bonaphthon), an antiviral agent,[107] from the corresponding 2-naphthol by oxidation with Fremy's salt, and studied its electrochemistry. Two reduction waves were observed for **11a** in DMF (Pt and HMDE). With platinum, the first, $E_{1/2}$ = −0.20 V (Table 3), was quasi-reversible and diffusion-controlled. ΔE_p increased from 70 to 110 mV upon a decade increase in the sweep rate (20–200 mV/s). The *CF* ratio of **11a** with benzil,[42] a compound known to undergo one-electron reduction, gave a value of 0.97 (Table 3), denoting the formation of the semiquinone. This intermediate shows good stability with an i_{pa}/i_{pc} value of 1.0 at 100 mV/s. The second wave (E_{pc} = −0.88 V) was broadened, reduced in height, and coupled to a small anodic peak, E_{pa} = −0.64 V. This behavior may be due to some type of chemical reaction after electroreduction,[111] such as proton abstraction from solvent. Very similar results were obtained with HMDE (Table 3).

An $E_{1/2}$ of −0.27 V is reported for the parent 1,2-naphthoquinone in DMF.[108] The effect of the bromo substituent is to make electroreduction more favorable. This is in accord with data for 2,3-dihydroxynaphthoquinone and its 6-bromo derivative.[110] The $E_{1/2}$ for the latter is 0.03 V more positive. Halogen in the 3-position should make the reduction potential more positive because of the inductive effect. Thus, 2-chloro-1,4-naphthoquinone reduces 24 mV more positive than the parent.[109] Bromo substitution exhibits similar influences.[110]

The proposed mechanism of oxidative stress for the *o*-naphthoquinone agents **11** is supported by several lines of evidence from closely related systems: (1) the *o*-naphthoquinone, β-lapachone (trypanocidal and antitumor agent),[5,112] shows activity linked to superoxide production and (2) activated oxygen species are formed by 1,2-naphthoquinone in rat liver microsomes.[113]

Phenols are widely used and represent one of the earliest classes of antimicrobial agents. The members

Table 3. Reduction Potentials and Electrochemical Characteristics of Naphthoquinones

Substrate	Electrode	Reduction Potential (V)	*CF* ratio[a]	i_a/i_c	Reference
11a[b]	Pt	−0.20[c]	0.97	1.0	—
11a[b]	HMDE	−0.20[c]	0.84	1.0	—
1,2-Naphthoquinone	—	−0.27	—	—	108
1,4-Naphthoquinone	—	+0.48	—	—	109
2-Chloro-1,4-naphthoquinone		+0.50	—	—	109
2,3-Dihydroxy-1,4-naphthoquinone	—	+0.29	—	—	110
6-Bromo-2,3-dihydroxy-1,4-naphthoquinone	—	+0.32	—	—	110

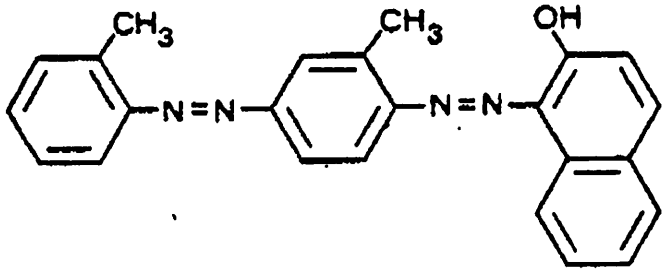
[a] See footnote *a* Table 1.
[b] 100 mV/s, tetraethylammonium perchlorate (0.1 M), substrate (0.5 mM), DMF, versus NHE.
[c] Quasi-reversible.

include mono- and diols that contain a wide variety of substituents.[32] A common transformation of phenols in vivo is hydroxylation to *o*- and *p*-diols followed by facile conversion to quinones.[84] Hence, it is conceivable that these types serve as precursors of quinones that function as the ultimate form of the drug via charge transfer.

Azo dyes and aromatic amines

Antibacterial activity has been observed with a number of azo dyes,[32] including scarlet red, 12, diacetazotol, and phenazopyridine.

Cyclic voltammetry was performed on 12 (scarlet red; Sudan IV) in DMF. The results are presented in Table 4. Compound 12 gave several reduction waves with $E_{pr} = -0.62$, -1.13, and -1.44 V, and subsequent oxidation waves at $E_{po} = -1.31$, -0.96, -0.67, and -0.33 V. The first wave, $E_{1/2} = -0.58$ V, is quasi-reversible and diffusion-controlled. ΔE_p ranged from 60 to 70 mV (scan rates of 50–200 mV/s) and was 65 mV at 100 mV/s. The i_{po}/i_{pc} value increased from 0.54 to 0.67 for the above scan rates. Slower scan rates (20 mV/s) produced no anodic peak. This indicates a reduction process accompanied by kinetic or other complications, such as fast follow-up chemistry. The *CF* ratio of 0.90 for 12 (benzil as reference)[42] denotes the transfer of one electron, with the formation



(12)

of the azo anion radical, in agreement with previous studies on various azo compounds in aprotic media.[114] The other waves were not examined in detail. Analogous results were observed with HMDE (Table 4) except that adsorption was found at scan rates of 50 and 20 mV/s. Upon the addition of acetic acid, reduction was observed at $E_p = -0.37$ V, accompanied by severe adsorption.

Other data on the reduction of azo compounds in aprotic solvents have been reported (in CH_3CN) (Table 4). The comparatively more positive value for 12 is expected because of increased conjugation. Previous studies have also been carried out in protic systems.[114] The $E_{1/2}$ values in buffered aqueous alcohol (C_2H_5OH or CH_3OH) for azobenzene, *p*-bisazobenzene, and 2-hydroxyazobenzene are presented in Table 4. Substituents on the aromatic nuclei; namely, methyl[115] and hydroxyl,[110] have an adverse effect on reduction as observed for the reported values.

A mechanism involving redox cycling resulting in oxidative pressure for the azo class is reasonable. It is postulated that the radical anion formed upon the incubation of sulfonazo III, a diazonaphthol, with hepatic microsomes reacts with oxygen in a catalytic manner, producing superoxide.[49] The azo radical anion intermediate was observed by ESR.

Alternatively, the active agent may be an aromatic amine generated by enzymatic reduction.[116] This type of transformation has been shown with 12 in rats.[117] A similar process may be responsible for the mutagenicity of Sudan IV, observed after chemical reduction and microsomal activation.[118] The most important members of the aromatic amine class contain a sulfonamide (sulfanilamide) or sulfone substituent on the nucleus in addition to the amino groups.[32,119] There is much documentation for the generally accepted view that the mode of action involves inhibition of dihydrofolate synthesis by competition with *p*-aminobenzoic acid. On the other hand, one should recognize that an appreciable body of data from studies on dapsone (*p,p'*-

Table 4. Electrochemical Characteristics of Azo compounds

Substrate	Electrode	Reduction Potential (V)	*CF* ratio[a]	i_{ox}/i_{red}	Reference
12[e]	Pt	−0.58[e]	0.90	0.61	—
12[f]	HMDE	−0.57[f]	0.82	0.58	—
Azobenzene	—	−1.12	—	—	114
Azobenzene	—	+0.352 − 0.079 pH[d]	—	—	114
1-Phenylazobenzene	—	−1.02	—	—	114
2-Methylazobenzene	—	−1.14	—	—	114
p-Bisazobenzene	—	+0.405 − 0.059 pH[e]	—	—	114
2-Hydroxyazobenzene	—	+0.29 − 0.068 pH[f]	—	—	114

[a] See footnote *a* Table 1.
[b] 100 mV/s, tetraethylammonium perchlorate (0.1 M), substrate (0.5 mM), DMF, versus NHE.
[c] Quasi-reversible.
[d] pH 1.6–9.0.
[e] pH 4.3–12.7.
[f] pH 2.0–6.0.

diaminodiphenylsulfone) points to the generation of reactive states of oxygen. A number of reports deal with in vivo or in vitro conversion to the *N*-hydroxy derivative[120,121] and possibly to the nitroso form.[120] Several investigations have demonstrated involvement of oxidative phenomena on exposure to dapsone,[120,122] including generation of superoxide and hydrogen peroxide by the hydroxylamino form in vitro.[121] Oxygen is required for the cytotoxicity observed during redox cycling with different xenobiotics, including aromatic amines.[123] The same intermediates generated by the partial oxidation of the amino functionality can also arise from the reduction of the nitro group.

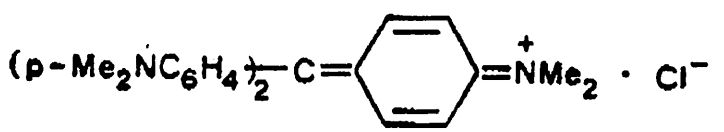
minium species

There are various subdivisions in this category, such as triarylmethane dyes, heterocyclic di-*N*-oxides, and heterocyclic salts. The first application of our working hypothesis to the mechanism of drug action involved the heterocyclic di-*N*-oxides.[20,21]

1. *Triarylmethane dyes*. Examples, all of which incorporate the iminium moiety, include gentian violet, and brilliant green. Compound **13** is used as a topical antibacterial, anthelminthic, and antifungal agent.[32] The synthetic dyes can produce toxic oxygen species through redox cycling.[112] The metabolism of gentian violet yields a one-electron reduction product, which was detected by ESR spectroscopy.[6] This delocalized radical reacts with oxygen to form a much more reactive peroxyl type. In 1952 the suggestion was made that the pharmaceutical effect of these drugs may be intimately associated with charge transfer.[124] The $E_{1/2}$ value for **13** is −0.55 V, and for brilliant green, −0.42 V. Some of the dyes are carcinogens, and gentian violet damages DNA.[6] This class, structurally related to quinones, represents one of the best supported examples of incorporation into our theoretical framework.

2. *Heterocyclic di-N-oxides*. Our initial investigation, experimentally based on the iminium **14** ET–oxy radical theory[22] included quinoxalines[20,21] and phenazines.[21] Representative members are dioxidine, **15**, and iodinin, **16**, which display $E_{1/2}$ values of −0.87 and −0.34 V (DMF), respectively. Reduction was favored by increased conjugation and intramolecular hydrogen bonding involving *N*-oxide. Reasonable correlations were found to exist involving reduction potential, structure, and drug activity for about 16 quinoxalines and 8 phenazines. The quinoxaline di-*N*-oxide free radical has been detected by ESR.[49] Other heterocyclic *N*-oxides may conceivably fit in this category.[125,126]

3. *Acridinium ions*. Proflavine and acriflavine are two of the more important representatives; occasionally quat salts are used.[32] Studies have demonstrated a correlation between base strength and antibacterial po-



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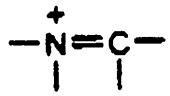

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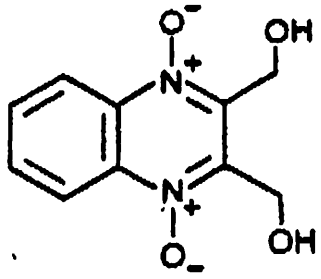
tency.[115] The acridinium cations that can arise at physiological pH are the active entities. These salts are necessary for strong binding with nucleic acid, as well as for bacteriostatic action. DNA intercalation is evidently associated with the biological effect. The $E_{1/2}$ value for 2,7-diaminoacridine is -0.59 V at pH 7.[126] Quite some time ago the proposal was advanced that these substances undergo in vivo reduction to radical anions that exert their antibacterial effect by interference with the respiratory electron transport chain.[126] Alternatively, we feel that reactive oxygen-containing entities may be generated, which could contribute to the toxic effect.

For the antimalarial drugs in this class,[15] namely, the 9-aminoacridine derivatives, oxidative stress and DNA strand cleavage are known to occur. Oxy radical formation is postulated to result from charge transfer by the heterocyclic iminium species. The reduction potential ($E_{1/2}$) of the quinacrine cation is -0.72 V (DMF), which is probably considerably more positive in vivo because of steric inhibition of resonance as a result of site binding. Kier, who suggested an ET mechanism, pointed out the favorable energetics associated with the protonated form during ET.[127]

4. *Quinolinium ions.* Illustrative of this type is quindecamine, 17, which has been used as a topical antibacterial and antifungal agent.[32] Clues concerning the possible involvement of an ET mechanism can be obtained by examining the literature[15] on closely related antimalarial agents, for example, chloroquine, 18a, and amodiaquine, 18b. The bases exist at physiological pH as cations that are involved in binding to DNA. Marked deviation of the side chain and nucleus from coplanarity is indicated, which should result in a shift of reduction potential to more positive values be-



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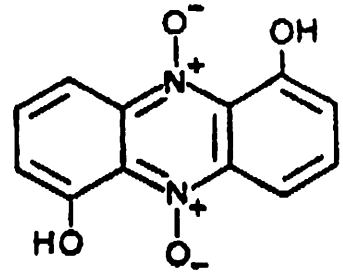

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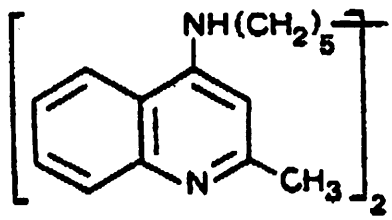
cause of steric inhibition of resonance in the cation. Free rotation of the side chain permits delocalization of the positive charge on the heterocyclic nitrogen making for greater difficulty in reduction. Electrochemical investigations have been carried out on a large number of quinolinium[14] and isoquinolinium[11] salts of varied structure that display anticancer properties. Reduction potentials fall in the range -0.43 to -1.5 V.

There is evidence for participation of reactive intermediates derived from oxygen for the 4-aminoquinolines.[15] Organic intercalating agents of the iminium type are known to photodamage DNA, presumably via activated oxygen.[128] The ribose unit was thought to be the electron donor.

5. *Other heterocyclic quat salts.* Bactericidal properties are exhibited by various compounds of this group, for example, benzo[h]naphthyridinium ions.[129] Pyocyanine, a natural *N*-methylphenazinium antibiotic, is quite susceptible to electroreduction,[130] $E_{1/2} = -0.54$ V. Mason has reviewed the chemistry of the *N*-methylphenazinium ion relative to the formation of radical cations and activated oxygen.[49] In the anticancer domain,[15] *N*-methylphenanthridinium salts are known to undergo charge transfer.[131] The activity of fused isoquinolinium salts has been correlated with the presence of the iminium site.[132] Binding to DNA could well be an important feature related to the activity of these types.

6. *From alkylating agents and DNA.* A considerable number of the gaseous chemosterilants are described as alkylating agents, including ethylene oxide,



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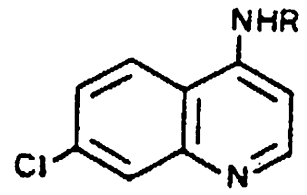
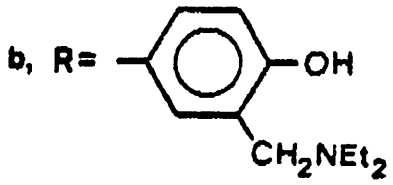


a, R = $-CH(CH_3)(CH_2)_3NEt_2$



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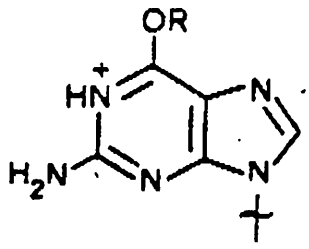
glycidaldehyde, aziridine, and β-propiolactone.[32] It is generally accepted that nonspecific alkylation of nucleophilic sites in essential metabolites constitutes the mode of action.

The alkylating class contains a large group of carcinogenic agents,[133] including those displaying antibacterial activity. Concomitant production of oxy radicals has been observed with various members.[4,7,134] Although the precise role of these reactive intermediates has not been ascertained, it appears that DNA strand cleavage may be a crucial event.[7,134] In a recent investigation of the mechanism of carcinogenesis, a novel proposal was advanced in which the salt form (iminium, 14) of alkylated nucleic acid was assigned a key function as an ET agent.[10] The purines (guanine and adenine) of DNA are the principal targets of attack.[133] For example, the ionic structure 19, a conjugated form of 14, is generated from O-6 alkylation of guanine and could conceivably undergo one-electron reduction. Electrochemical data from the literature[135] and our own studies[10] are in reasonable accord with the current picture relating site of alkylation and defect persistence to oncogenic response. Thus, it appears quite plausible that the salt form is functioning in a



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catalytic manner as a generator of toxic oxy radicals. A similar rationale applied to the sterilant types would incorporate them into the generalized ET–oxy radical approach.

Naturally occurring antibiotics

A number of naturally occurring antibiotics[32] fall into the structural categories that have been discussed, for example, albomycin (Fe chelate), ferrimycin A, (Fe chelate), bleomycin (Fe or Cu chelate), lucensomycin (epoxide), oleandomycin (epoxide), chloramphenicol ($ArNO_2$), pyrrolnitrin ($ArNO_2$), rifamycin (quinone), geldamycin (quinone), actinomycin D (iminoquinone), tetracyclines (possible precursor of iminoquinone[15] or metal chelate[89]), streptovaricins (*p*-quinone monomethane), CC-1065 (alkylating agent),[136] kojic acid (metal chelate),[89] and bacitracin (metal chelate).[89]

Other considerations

In our prior discussion, much evidence has been cited for the formation of radical intermediates and involvement of activated oxygen species. Superoxide is presumed to be the precursor. Increasing evidence from studies involving xenobiotics and the initiation and progression of various diseases implicates free radicals derived from oxygen.[1-9,35,137]

According to the theory, the different agents act in vivo as ET entities in the production of oxy radicals or disruption of normal ET. However, some of the reductions were found to be irreversible. Reversibility may be affected by the scan rate employed, as observed in this and other studies.[138] Association with the active site resulting in immobilization could prevent reactions which otherwise occur in vitro, such as dimerization, thus making ET possible. This aspect has been treated elsewhere.[14,17,21] Several reports indicate that reduction potential in vivo may well be better than in vitro.[124,139] Evidence points to a relationship between electrochemical characteristics of drugs and physiological activity. For example, antibacterial mitomycins (quinones) possessing less negative $E_{1/2}$ values exhibited more powerful activity.[140] A study on the antibacterial activity and electrochemical activity of 9-methylaminoacridines and their nitro derivatives revealed a symbatic relationship.[141] Similar correlations have been noted for heterocyclic di-*N*-oxides[20] and heterocyclic nitro compounds.[44,46,142] Other examples are given elsewhere.[10,16]

The present theory is clearly an oversimplification of a very complex situation, since absolute correlation between electrochemical behavior and physiological

(20)

activity is not expected because of the many variables involved in vivo, for example, metabolism, stereochemistry, cell permeability, solubility, site binding, and diffusion. It should be emphasized that the toxic effects of antibacterial agents may be manifested by a variety of routes. One of the most widespread and generally accepted mechanistically is interference with DNA replication or synthesis.[32] For aliphatic quaternary ammonium salts, such as cetrimonium bromide, 20, and extended-chain imidazolium[143] and pyridinium salts,[32,143] cell membranes and closely associated structures are likely targets.[32] Conceivably a number of pathways, including oxy radical generation, may act in concert for certain drugs.

Examination of the literature reveals that many of the classes discussed here also display activity in other medicinal areas.[105] Furthermore, large numbers of drugs elicit carcinogenic,[4,35,49,144,145] mutagenic,[4,32,44,49,144,145] cardiotoxic,[137] and pesticidal[61] responses. Since we are stressing a unifying theme, it might well be that the other effects are frequently related to ET–oxy radical involvement.

In summary, based on the evidence from prior contributions and our work, the various categories appear to display the following common characteristics: binding to DNA, involvement in ET, and production of activated oxygen. However, there are gaps for the various categories, which need to be filled by further investigation.

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